RAPID AMPLIFICATION OF cDNA ENDS AND cDNA SCREENING OF *ALCOHOL DEHYRDROGENASE* GENES FROM *METROXYLON SAGU*

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ABBREVIATIONS

Adh	Alcohol dehydrogenase gene
BLAST	Basic Local Alignment and Search Tools
cDNA	Complementary DNA
CTAB	Cetryl trimethyl ammonium bromide
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylene diamine tetraacetic acid
EtBr	Ethidium bromide
GSP	Gene specific primer
LiCl	Lithium chloride
$MgCl_2$	Magnesium chloride
M-MuLV	Moloney murine leukemia virus
NaAc	Sodium Acetate
NADH	Nicotinamide adenine dinucleotide
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
RNase	Ribonulease
RT	Reverse Transcriptase
TAE	Tris acetate EDTA

Rapid Amplification of cDNA Ends and cDNA screening of *Alcohol dehydrogenase* Genes from *Metroxylon sagu*

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ABSTRACT

Metroxylon sagu is one of the most important crops that found abundantly in Sarawak especially in freshwater swamp area where it facing environmental stress such as flooding that causing metabolisms changing in the cell. One of the important gene encodes during this stress period is *alcohol dehydrogenase* (*Adh*) gene which involve in fermentation metabolism in attempt to produce energy without presence of oxygen. The objective of this study was to isolated full length of *Adh* gene through RACE. RNA from waterlogged roots and leaves of *M. sagu* was extracted and first strand cDNA was develop form it and then undergo PCR process with using specific primer in attempt to extract *Adh* gene. Combination of ADH primer 1 and 3' inner primer produced reliable PCR product which undergo gel extraction to obtain pure product which subsequently sent for DNA sequencing purpose. The result was not the desired *Adh* gene due to BLAST search and several other attempt was done in screening for *Adh* gene using other primers. Only screening done by using combination of primer PMB Adh1-f/r gives PCR product gave result at 250 bp at range 40-50^{o}C.

Keywords: *Alcohol dehydrogenase* (*Adh*) genes, *Metroxylon sagu*, RACE, PCR,

ABSTRAK

Metroxylon sagu adalah tanaman penting yang banyak ditemui di Sarawak terutamanya di kawasan paya air tawar di mana ia menghadapi tekanan persekitaran seperti banjir yang menyebabkan perubahan pada metabolisme pada sel. Salah satu gen penting yang di ekspreskan semasa menghadapi tekanan ialah gen alcohol dehydrogenase (Adh) yang terlibat dalam metabolisme fermentasi dalam usaha menghasilkan tenaga tanpa kehadiran oksigen. Matlamat kajian ini adalah untuk mengasingkan gen Adh penuh melalui RACE. RNA daripada akar yang terendam dalam air dan daun dari M. sagu telah dikeluarkan dan rantaian pertama cDNA dibuat dari nya dan melalui proses amplifikasi DNA (PCR) dengan menggunakan primer yang khusus dalam usaha mengasingkan gen Adh. Kombinasi primer ADH primer 1 dengan 3' inner primer menghasilkan produk PCR yang baik dan melalui pengasingan gel bagi mendapatkan produk tulen yang kemudiannya dihantar untuk proses penjujukan DNA. Hasil dari penjujukan ini dan carian BLAST telah menunjuk bahawa jujukan tersebut bukan gen Adh dan dengan itu, cubaan lain dibuat bagi mencari gen Adh menggunakan primer lain. Produk PCR bersaiz 250 bp dihasilkan pada suhu antara 40-50^{o}C yang baik ditunjukkan oleh kombinasi primer PMB Adh1-f/r.

Kata kunci: Gen alcohol dehydrogenase (Adh), Metroxylon sagu, RACE, PCR,

CHAPTER 1

INTRODUCTION

1.1 Introduction

Plant use in this study was *Metroxylon sagu* Rottb., found many in Malaysia especially in Sarawak. It is believed to be indigenous at Indonesia (Irian Jaya and Moluccas) and Papua New Guinea, introduced to Malaysia and Philippines (Wiki, 2007). Plant part used in this study was its leaves, obtained from Universiti Malaysia Sarawak's nursery, and waterlogged roots, obtained from Genetic Engineering Lab's Master degree student. The purpose of using leaves as sample was to detect the presence of *alcohol dehydrogenase* (*Adh*) gene while waterlogged roots used because of the stress that the root faced which may induced the production of *Adh*.

Adh is an important gene that being expressed by plant due to the stress condition. Purpose of this study was to extract full length of *Adh* gene by using Rapid Amplification of cDNA Ends (RACE) protocol. Drew (1997) confirmed that waterlogged root that suffered from declining of oxygen supply, subsequently reduce in ATP/ADP ratio will enter fermentation phase to slowly produce ATP with ethanol as final product of pyruvate. Thus, this project focused more to those sample compared to other part of *M.sagu* tree.

To detect the presence of *Adh* gene in the sample, total RNA was extracted which then converted into first strand, enable it to undergo PCR process with several pairs of primer. These primers helped in producing complement DNA sequence that probably desired *Adh* gene. Specific PCR product then sent for sequencing and then BLAST search being done to confirm the obtained DNA.

1.2 Statement of problems

M. sagu is one of the plant that able to tolerate during flooding period which may produce *Adh* gene that involve in anaerobic metabolism to produce ATP but this point still not strong fact since the full length of *Adh* has yet to be isolate from *M. sagu*. Screening and optimization of PCR using several primers and changes in $MgCl_2$ concentration and temperature help in detecting *Adh* gene.

1.3 Objectives

The objective of this study is to isolate full length of *Adh* gene and detecting the presence of it the waterlogged roots and leaves of *M. sagu*. Optimization of PCR is important in producing better DNA band for DNA sequencing in confirming the band either *Adh* gene or not. Another objective is to screening *Adh* gene by using several primer for further research and change the $MgCl_2$ concentration that help in enhancing the primer attachment on the template help in producing more reliable band that can be isolate and send for sequencing.

CHAPTER 2

LITERATURE REVIEW

2.1 *Metroxylon sagu*

M.sagu is the plant that is important for economical purpose in Malaysia, abundant and commercialize in Sarawak state. This plant live in the freshwater swamp area which is waterlogging tolerate and non-tolerate to water shortage which may stunning its growth (McClatchey *et al.*, 2006). In Sarawak, *M.sagu* cultivated mostly in Mukah area and its common name among indigenous Melanau people is *balau* and *rumbia* in Malaysian national language.

According to McClatchey *et al.* (2006), *M. sagu* life cycle is about 12 years, divided into several stage of development; (i) Rosette formation in 45 months, (ii) Bole formation in 54 months, (iii) inflorescence in 12 months, and (iv) fruit ripening in 24 months. This plant harvested to obtain sago starch that stored in its stem and for many cases, it is harvested before the plant start to flowering where farmer detect this period by observing inflorescence development (McClatchey *et al.*, 2006). Traditional cultivation area in the natural freshwater swamp by indigenous Melanau people shows a shortening of harvesting time to only 7 years compare to the research done by The Crop Research and Application Unit (CRAUN) Sarawak which take up to 8 years before harvesting period. This is because *M. sagu* is shade tolerant plant which confirm by McClatchey *et al.* (2006) which describe that *M.sagu* acquired 50% of shade, found most in normal canopy of plant in swamp area rather than open area that give almost 100% of sunlight that may slow the growth process.

M. sagu starch that stored in the stem can be eaten raw and also can be extract out to produce pure sago starch which then converted into sago flour, bread, pudding and special cuisine that only can be found in Sarawak is sago pearl or local name known as *sagon*. Research done by Wong *et al.* (2007) found that enzyme pullulanase act as debranching agent to sago starch to produce linear long-chain dextrin (soluble gummy use as food thickening agent) up to 32.9% from total mass after 18 hours treatment. This will help in diverse the usage of sago starch for increasing demand of it which will generate income for Malaysian economy.

University Malaysia Sarawak is one of the most leading expert that have done many research on utilizing sago product to produce other product such as lactate production using *Lactococcus lactis* (Bujang *et al.*, 2002) and ethanol production using *Zymomonas mobilis* (Bujang *et al.*, 2000). Research on obtaining high production rate condition are on progress which maximizing the product.

2.2 Anaerobic Respiration

Anaerobic is define as a condition where oxygen is lack or none at all due to the increasing of oxygen demand to supply ratio which forces cells to enter anaerobic respiration or known as fermentation metabolism. Janiesch (1991) shows that 5-24 gram oxygen consumes by plant roots and microorganisms per square land. Flooded condition restricted the oxygen diffusion to reach submerged roots, subsequently initiate anaerobic condition who forces the cell to enter anaerobic respiration. This is because oxygen vital for glucose breakdown to carbon dioxide and water where 38mol of ATP produce for each mol of glucose.

The absence of oxygen will cause blockage of electron transport chain in mitochondria because transfer of electrons from cytochrome oxidase (complex IV) to oxygen cannot take place, resulting in accumulation of N*ADH*+H^+, product of citric acid cycle. The ratio between N*ADH*+H^+/ NAD^+ will getting higher which will inhibit the citric acid cycle itself. Anaerobic respiration start to reduce number of N*ADH*+H^+ by pyruvate converted into acetaldehyde catalyst by enzyme pyruvate decarboxylase and *ADH* enzyme help in converting acetaldehyde and N*ADH*+H^+ to ethanol and NAD^+.

$$\text{Pyruvate} \xrightarrow[\text{pyruvate decarboxylase}]{} \text{Acetaldehyde}$$

$$\text{Acetaldehyde} + N\mathit{ADH} + H^+ \xrightarrow[\text{alcohol dehydrogenase}]{} \text{Ethanol} + NAD^+$$

Higher plant such as *M.sagu* has developed several ways in order to overcome flooding stress, as described by Janiesch (1991) which assist the plant to survive in range from several hours to several months, vary on the plant species; (i) ATP production via fermentation process, (ii) produce non-toxic end product and (iii) transport of oxygen from atmosphere to the cell. Drew (2007) also shows the transfer of ATP from nearby normoxic cells.

2.3 Normoxia, hypoxia and anoxia

Normal condition with adequate oxygen concentration is called normoxia such as most leaves who expose to air whole time and the source of ATP mainly produce by normal glucose breakdown to carbon dioxide and water. Hypoxia meanwhile is the transition state between normoxia and anoxia where both of respirations running at the same time as described by Saglio *et al.* (1999). It is commonly occurred in the lowland area which prone to have flood and the inner part of stem cells which reduce more than 50% of total oxygen available in the air (Drew, 2007). Anoxia is the condition where oxygen is totally absence and the only source of energy is came from anaerobic respiration process (Saglio *et al.*, 1999). Drew (2007) state that anoxia condition may killed the cell, commonly cause by high number of acid that reduce dramatically cytoplasm pH.

Acid that being produce in *Adh1* mutant maize is lactic acid, product of lactate dehydrogenase which then reduced cytoplasmic pH which confirms that wild-type are able to maintain cytoplasmic pH in anoxia condition (Drew, 2007). It shows that desired *Adh* gene transcribe during hypoxia and anoxia enable waterlogged roots of *M.sagu* is the best source to get it. Study by Ratcliffe (1995) shows that expression of *Adh* gene greater in hypoxia condition compare to anoxia

2.4 *Alcohol dehydrogenase* gene

Adh gene is important gene that assist seedling development, fruit ripening, and pollen development (Randall, 2000). But, the most important function in higher plant is helping in facing stress especially in hypoxia and anoxia condition, supported by Morton *et al.* (1996) who shows that *Adh* promoter transcription rate higher in both oxygen stress and cold stress. An *Adh*-null mutant shows that they do not survive 24 hours of anoxia (Saglio, 1999). The cell death for *Adh*-null mutant or anoxia non-tolerate plant cause by the dramatic pH declining result in accumulation of lactic acid produce by lactate dehydrogenase where *Adh* can produce less toxic product which is ethanol.

Two or three *Adh* isozymes observe in all flowering plant and according to Morton et al 1996, from a representative of Arecaceae (palm family), *Washingtonia robusta*, this species may be contain three functional *Adh* loci. Thus, for the purpose of extracting *Adh* mRNA from the sample, it is better to use the sample that undergo certain condition such as anoxia and hypoxia which have a higher level of *Adh* mRNA compare to the plant at the normal state.

2.5 Rapid amplification of cDNA ends (RACE)

RACE is a technique that able to generate full length of desire gene by using normal PCR machine with a very specific primer which complement to it. Total RNA are required to develop cDNA because eukaryotes RNA contain poly(A) tail which then use to complement with poly(T) sequence in the first primer called Q_T primer. Then, reverse transcriptase enzymes will generate new complementary DNA sequence based on the RNA sequences before that provide the first cDNA template that crucial for PCR technique since RNA will degrade due to the high temperature of PCR technique. This cDNA first strand will undergo normal PCR by using a specific primer call Gene Specific Primer (GSP) + Q_1 primer. With this GSP, other genes than *Adh* will never been generate because GSP only attach at a very specific site on *Adh* gene.

CHAPTER 3

MATERIALS AND METHODS

3.1 Plant materials

Leaves and waterlogged roots were *M. sagu* part that used in this research. Leaves used were specifically to young leaves that obtained from Universiti Malaysia Sarawak's nursery located at the east campus. It then rinsed through running water and treated with 70% ethanol to reduce contamination by microbes. It is then preserved at -80^{o}C to retain it's contain especially RNA contain. While for waterlogged roots was obtained from Genetic Engineering Lab's Master Degree student, that preserved also in -80^{o}C.

3.2 Total RNA Extraction

There are two types of RNA extraction done in this project, modified from methods by Gasic *et al.* (2004), large scale and small scale. Small scale extraction was purposely to extract only small RNA quantity which takes place inside 1.5 ml eppendorf tube while large scale can maximize the RNA extraction 10 times of quantity compare to small scale, take place in 50 ml falcon tube.

Sufficient quantity of *Metroxylon sagu* (either roots or leaves) was grinded in mortar with liquid nitrogen. Grind sample then put inside 1.5 ml eppendorf tube (small scale) or falcon tube 50 ml which extraction buffer (appendices) then added in the tube 700 µl for small scale while 7 ml for large scale (prewarm at 65^{o}C). The mixture then vortex into single phase and incubate at 65^{o}C for 45 minutes. During the incubation, the mixture was vortex into single phase periodically for 2-3 times to avoid any coagulations of the plant sample and increase the digestion rate by

extraction buffer to the sample. Then, equal volume of Chloroform:Isoamyl (Mallinckrodt Baker) (24:1) added into the mixture which then undergo centrifugation at 13,000 rpm for 15 minutes at 4^{o}C.

Supernatant then transferred to new tubes. For large scale, supernatant then transferred from falcon tube to 10 of 1.5ml of eppendorf tubes which make it the same as the small scale process where each may consist of 500-700 µl supernatant. Then, equal volume of Chloroform:Isoamyl (24:1) added for second time of centrifugation at 13,000 rpm for 15minutes at 4^{o}C. The final supernatant then transferred into new tube and added with 1/3 volume of 8.0 LiCl (MP Biomedicals Inc.) and incubate at -20^{o}C overnight.

Day 2 needs the tube to undergo centrifugation at 13,000rpm for 15minutes at 4^{o}C, purposely to pellet the residue. Then, the pellet needs to wash with 500µl 70% ethanol (v/v) and let it air dry before being added 20µl of 3M NaAc (pH 5.2) (Sigma) and 500µl 70% ethanol (v/v). The mixture then incubated overnight or 3hours in -80^{o}C before enter the next step.

Next step/day 3 also required the centrifugation at 13,000rpm for 15minutes at 4^{o}C for pellet the residue. The pellet then washed by 70% ethanol and let it air dry before dissolved the pellet (crude RNA) in DEPC treated water range 30-50µl, depending the size of the pellet which indicate the amount of crude RNA. Then, this crude RNA can be stored at -80^{o}C for further use.

3.3 DNase Treatment

DNase enzyme (Fermentas) treatment was done either according to manufacturer's recommendation or modification as following; for samples that has high concentration of DNA, modification was done by increasing 1u/µl DNaseI volume to 1.5 µl and decreasing RNA volume to 7.5 µl. This is to maintain the final concentration of 1 µl 10X reaction buffer into 1X and increasing the rate of DNA digestion by DNaseI during 37^{o}C incubation for 30 minutes. 1 µl of stop solution (25 mM EDTA) was added to stop the reaction followed by incubation at 65^{o}C for 10 minutes.

3.4 First strand cDNA synthesis

First strand synthesis was done by using reverse transcriptase (RT) enzyme (Fermentas) either according to manufacturer's recommendation or modification as following; for low concentration of RNA in the sample, normal volume of DNase treated RNA increased from 5 to 10 µl in order to provide more RNA for RT process. The amount of 200u/µl RevertAid^{TM} M-MuLV RT (Fermentas) increased from 200 units to 300 units to increase the RT activity. Primer use in first strand synthesis was 3' adapter primer (5'-GCG AGC ACA GAA TTA ATA CGA CTC ACT ATA GTG TTT TTT TTT TTT TTV N-3') (First Choice® RLM-RACE Ambion)

3.5 Polymerase Chain Reaction

PCR mixture was done with 3 different PCR components either from Qiagen, Fermentas, or Vivantis either followed protocol provided by manufacturer or modified as described: optimization of $MgCl_2$ was made by addition of 25 mM of $MgCl_2$ into mixture that have final volume of 25 µl where each 1 µl represent each 1 mM $MgCl_2$ needed. For *Taq* PCR Master Mix (Qiagen) was only needed to undergo dilution from 2X to 1X solution and the $MgCl_2$ optimization done by addition of 25 mM of $MgCl_2$ into master mix which already have 1.5 mM of $MgCl_2$. The amount of template volume also increased from 1 µl to 2 µl when the template was low in concentration to ensure the PCR activity in running. The concentration of *Taq* polymerase increased from 1 unit to 2 units per reaction to increase its activity, thus produce a better PCR product with high concentration. The parameter use in PCR described as in table as followed;

Table 3.1: Parameter for PCR cycle

Steps		Temperature (^oC)	Time (min)
Step 1	Initial Denaturation	94	3
Step 2	Denaturation	94	1
Step 3	Annealing	*x*	1
Step 4	Extension	72	1
Step 5	Repeating Step 2 – 4 for 35 cycles		
Step 6	Final Extension	72	7

Primer used in this study listed in the following table;

Table 3.2: Primer used in this study with annealing temperature

Primer name	**Sequence**	**Annealing Temperature (*x*)* ^{0}C**	**References**
ef1 f	5'-ATT GGA AAC GGA TAT GCT CCA-3'	56	Nicot, 2005
ef1 r	5'-TCC TTA CCT GAA CGC CTG TCA-3'	56	Nicot, 2005
3' Outer Primer	5'-GCG AGC ACA GAA TTA ATA CGA CT-3'	56	First Choice® RLM-RACE Ambion
3' Inner Primer	5'-CGC GGA TCC GAA TTA ATA CGA CTC ACT ATA GG-3'	50	First Choice® RLM-RACE Ambion
ADH_Primer1	5'-AGG GAT CCT YTG CCA CAC HGA TGT KTA CTT CTG GGA-3'	Depending on either 3' inner or 3' outer primer	Roslan, Personal communication
Adh1ha-f	5'-CAT GTC CTT CCT GTG TTC AC-3'	N/A	Roslan, Personal communication
Adh1ha-r	5'-TGC GGA TGA TGC AGC GGA T-3'	N/A	Roslan, Personal communication
PMB Adh1-f	5'-GTN GGN GAR GTN CAN GA-3'	N/A	N/A
PMB Adh1- r	5'-TTY CAY TAY ATH CCN AA-3'	N/A	N/A
haADH-f	5'-TAC TTC TGG GAA GCC AAG GGA CAA-3'	N/A	Roslan, Personal communication
haADH-r2	5'-CTC AGC AAT CAC CTC TTC AA-3'	N/A	Roslan, Personal communication
WASro Adh-f	5'-GGG TGC TGT AGG CCT TGC-3'	N/A	Morton, 1996
WASro Adh-r	5'-GAT ATC TGC ATT TGA ATG CG-3'	N/A	Morton, 1996

Mixed bases nomenclature:
R=A/G, M=A/C, W=A/T, H=A/T/C, V=G/A/C, D=G/A/T, Y=C/T, K=G/T, S=G/C, B=G/T/C, N=A/C/T/G
* N/A- not available

The non available annealing temperature for certain primers were due to the non-optimize primer which needs further screening and optimization in order to get better band from *M. sagu* sample.

CHAPTER 4

RESULTS & DISCUSSION

4.1 RNA extraction

Several attempted in order to extract RNA from both waterlogged roots and leaves from *M. sagu* until the most successful attempt was shown as following figure. This RNA extraction was using small scale method that give not a very satisfactory result since the concentration based on the brightness of RNA bands are not to good. This may cause by the uneven distribution of grind *M. sagu* sample into the tube that give produce uneven RNA extracted out from it. The first step in putting grind sample into the tube required faster work since broken cell in the grind sample exposing RNA to the air subsequently to the oxygen which may oxidize them.

Figure 4.1 shows the result of RNA extraction from *M. sagu* leaves. There are several faded band which can be assume to have very little concentration of RNA which needs several modification in downstream process in order to get good result. Tube 10 give no result at all probably cause by too little of sample being put (maybe it is the last tube that get the sample) or the RNA is already oxidize by oxygen in the air.

Waterlogged roots of *M. sagu* done by using large scale methods, shown by Figure 4.2. The result shows almost equal amount of RNA with bright band appeared on the gel electrophoresis. This is because of the large scale was used gave better amount of RNA extracted and equally distribution of RNA compare to the small scale because the distribution occur in liquid form of digested sample compare physically distribution in small scale. In large scale, faster transfer of grind sample into tube containing extraction buffer gave advantage of less oxidation occurred by the presence of β-mecarptoethanol (BDH Laboratory). Since the tube is

bigger (50ml), more grind sample can be put together result in more concentrated RNA produced at the end of the process and it is crucial for downstream process.

Incubation of overnight between day one and day two in 8.0 LiCl (MP Biomedicals Inc.) that is less then the time of LiCl addition gave better RNA band compare to the incubation that more than 24 hours. While incubation in 3M NaAc (pH 5.2) (Sigma) can withstand more than 24 hours and gave better RNA band thus if incubated in 24 – 48 hours time period.